5.1. INTRODUCTION

Once the isolation and purification processes of the cyanobacteria have been accomplished, it is important to maintain and preserve the cultures in viable condition for future references and research activities. Generally, cyanobacteria are maintained by subculturing them in BG-11 broth or on agar slants. The maintenance of cyanobacterial cultures usually required propagating working culture by subculture and keeping alive in growing condition. Immobilization can be carried out either by physical means such as adsorption or entrapment of the cells in a gel or foam matrix or by chemical methods such as covalent binding. The advantages of immobilization are: stabilization of the catalytic activity resulting in increased product formation, ease of its separation from the medium and re-use of catalysts for extended periods of time.

The primary purpose of preserving cultures is to maintain cyanobacterial population in a viable state for considerably longer period. During preservation all physiological processes of an organism were considerably slowed down, without affecting viability. One possibility for avoiding internal freezing would be the addition of cryoprotectants just above the freezing temperature prior to chemical fixation before super cooling (Withers, 1985). Indeed, cryoprotectants have colligative properties and maintain water in the liquid state by forming hydrogen bonds, preventing the diffusion of water molecules to the ice front and participating in the crystallization process (Finkle et al., 1985). A preserved culture can be reactivated whenever desired by providing suitable growth conditions. Many researchers have isolated the cyanobacterial strains for the different studies and lost most of them after the studies were finished because of several factors like lack of proper preservation method,
manpower and equipments such as a controlled cooling apparatus and liquid nitrogen chamber. This has resulted to a wasted effort of isolation and loss of research materials. Therefore, proper maintenance and preservation which is an essential requisite for fruitful research has been adopted for the selected cultures showing high nitrogenase activity using cryoprotectant DMSO and cell immobilization with storage condition at 18±2°C would be executed for future studies.

5.2. MATERIALS AND METHODS

5.2.1. Maintenance in liquid (broth) medium and agar slants medium

5.2.1.1. Preparation of broth and slants

60 ml BG-11 (-N) broth was dispensed in 100 ml conical flask, cotton plugged and autoclaved at 15 psi for 15 mins at 121°C. For slants BG-11 (-N) agar medium was prepared and dispensed 5-6 ml in culture tubes, cotton plugged and autoclaved at 15 psi for 15 mins at 121°C.

5.2.2. Cell immobilization (Musgrave et al., 1982)

5.2.2.1. Preparation of stock solution and inoculation

Sodium alginate solution 1.5% (w/v) was prepared in BG-11 (-N) medium by warming the solution in a water bath. 3 ml of 15 days old culture was added after the solution cooled down to room temperature. The solution was mixed thoroughly and using a micropipette, the mixture was added drop wise into 100 ml of 1% CaCl₂ solution in a laminar flow cabinet. Calcium alginate beads (~2 mm diameter size) formed in the CaCl₂ solution was left in the same solution for hardening at 4°C for 1 h. The beads were harvested and washed with sterile distilled water and in BG-11 (-N) medium.
5.2.2.2. Processing of the samples

The immobilized beads were transferred to 15 ml screw capped tubes containing BG-11 (-N) broth medium and stored at 18±2°C under photon flux rate of around 20 µmol m⁻² s⁻¹ and at 4°C.

5.2.2.3. Revival of the samples

The immobilized beads (5-10 approximately) were inoculated into 50 ml of fresh BG-11 (-N) medium in 100 ml conical flask and incubated at 28±2°C under continuous illumination of 54-67 µmol m⁻² s⁻¹ light intensity.

5.2.3. Cryopreservation (Day and Brand, 2005)

5.2.3.1. Preparation of stock solution

Stock solution A:

16% (v/v = volume per unit) DMSO (Dimethylsulphoxide 99.9%) in BG-11 (-N) broth

Stock solution B:

20% (v/v) Glycerol in BG-11 (-N) broth

The above solutions were filtered by syringe filter (Axiva) and stored in fridge at 4°C temperature.

5.2.3.2. Inoculation of the samples

i) 900 µl cyanobacterial suspension was transferred in each vials containing 900 µl of each stock.

ii) Volume of each cryovials: 900 µl solution and 900 µl cyanobacterial suspension i.e. 1.8 ml

iii) Cultures were preserved in triplicates.

iv) 1.8 ml total volume was diluted to 50% as due to inoculation of cyanobacterial suspension in equal amount into stock solution.
5.2.3.3. **Processing of the samples**

The samples in cryovials were stored at -60°C deep freezer in dark condition.

5.2.3.4. **Revival of the samples**

1. The cryopreserved samples were taken out from -60°C (for both the two solutions i.e. DMSO and Glycerol).

2. The cryovials were thawed at 35°C in 20-30 ml distilled water (in beaker) for 3-4 mins (until ice melts completely and the contents of the cryovials warmed to near room temperature) but not above room temperature.

3. The samples were centrifuged for 3 mins at 3000 rpm in a centrifuge to pellet the culture as too much centrifugation can lower the viability.

4. The supernatant was discarded and 1.8 ml sterilized BG-11 (-N) broth was added.

5. The sample was centrifuged for 3 mins at 3000 rpm.

6. The supernatant was discarded and 1.8 ml sterilized BG-11 (-N) broth was added again and centrifuged the sample for 3 mins at 3000 rpm.

7. The culture pellets were transferred from cryovials into 50 ml BG-11 (-N) broth with proper labelling.

8. The flask was incubated by covering with black cloth for 1-2 h in culture room.

9. The black cloth was removed and allowed growing the cultures in normal culture conditions at 28±2°C under continuous illumination of 54-67 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) light intensity.

10. After one week, revival was checked both by visibility and microscopic observation.

    * Estimation of chlorophyll-a for checking of successful revival rate for both the methods adopted was carried out as described by Mckinney (1941).

5.2.4. **Statistical analysis**

Standard deviation of the data was calculated using Microsoft office Excel 2007.
5.3. RESULTS

All the 247 Nostoclean cyanobacteria which belong to 12 genera were maintained in BG-11 (-N) agar slant quadruplicate tubes and separately one BG-11 (-N) broth (photoplate-22). Out of these, 06 strains which showed high nitrogenase activities were subjected for cell immobilization and cryopreservation for future study without losing their viability. The viability level after six months of preservation has been shown (photoplate-23). The post-thaw viability varied depending on the preservation methods. The selected strains comprised of Anabaena sp. BTA650, Nostoc sp. BTA197, Rivularia sp. BTA510, Scytonema hofmanni BTA124, Anabaena sp. BTA281 and Calothrix sp. BTA265 were revived completely after six months of cryopreservation (fig.9) and immobilization (fig.10). In case of cryopreservation with cryoprotectant DMSO, all the 06 strains were revived fairly well in one to two weeks time and yielded chlorophyll-a as follows: Nostoc sp. BTA197 (9.76±0.17 µg ml⁻¹), Anabaena sp. BTA650 (7.91±2.15 µg ml⁻¹), Scytonema hofmanni BTA124 (5.71±0.44 µg ml⁻¹), Anabaena sp. BTA281 (2.12±0.15 µg ml⁻¹), Rivularia sp. BTA510 (1.90±0.12 µg ml⁻¹) and Calothrix sp. BTA265 (1.29±0.23 µg ml⁻¹).

With cryoprotectant glycerol, comparatively all the 06 strains performed poorly under same conditions and time period but revival was observed in all of them. Chlorophyll-a estimation result shown were Scytonema hofmanni BTA124 (4.27±0.18 µg ml⁻¹), Anabaena sp. BTA281 (2.92±0.13 µg ml⁻¹), Anabaena sp. BTA650 (2.14±0.24 µg ml⁻¹), Rivularia sp. BTA510 (2.12±0.21 µg ml⁻¹), Nostoc sp. BTA197 (1.82±0.13 µg ml⁻¹) and Calothrix sp. BTA265 (1.14±0.12 µg ml⁻¹). For the immobilization technique, all the 06 strains at 18±2°C under photon flux rate of around 20 µmol m⁻² s⁻¹ revived very exuberantly but at 4°C Calothrix sp. BTA265 and Rivularia sp. BTA510 showed no revival inspite of subjection to the same culture conditions. Under storage condition of 18±2°C, Scytonema hofmanni BTA124 yielded the highest chl-a (14.95±1.66 µg ml⁻¹) followed by Nostoc sp. BTA197
Fig. 9: Chl-a (mean±SD) after revival of cultures from cryopreservation using different cryoprotectants.

Fig. 10: Chl-a (mean±SD) after revival of cultures from cell immobilization.
(13.29±2.19 µg ml\(^{-1}\)), Anabaena sp. BTA650 (6.44±0.17 µg ml\(^{-1}\)), Rivularia sp. BTA510 (5.37±0.99 µg ml\(^{-1}\)), Calothrix sp. BTA265 (2.38±0.22 µg ml\(^{-1}\)) and Anabaena sp. BTA281 (2.10±0.03 µg ml\(^{-1}\)). Under storage condition of 4°C, Scytonema hofmanni BTA124 yielded the highest chl-a (8.87±0.49 µg ml\(^{-1}\)), followed by Nostoc sp. BTA197 (1.89±0.37 µg ml\(^{-1}\)), Anabaena sp. BTA281 (1.88±0.18 µg ml\(^{-1}\)) and Anabaena sp. BTA650 (0.32±0.03 µg ml\(^{-1}\)).

Results were expressed as mean±SD of three independent replicates.

5.4. DISCUSSION

Compared to continuous subculturing in liquid medium, growing them in slants has proven useful for all the genera. Usually, maintenance of working culture required the periodic transfer of strain to fresh minimal agar medium. The strains were maintained in very low light intensity of 20 µmol m\(^{-2}\) s\(^{-1}\). They were streaked on cotton-plugged agar slants and allowed to grow. Frequency of sub culturing must be kept to a minimum, but it has been separately determined for each organism (Kaushik, 1987). Cryopreservation using cryoprotectant DMSO seems to be a better option than glycerol though the revival in glycerol cannot be ruled out totally. All the different strains which belong to different category of Nostocales showed high revival rate in DMSO. For immobilization, temperature 18±2°C under photon flux rate of around 20 µmol m\(^{-2}\) s\(^{-1}\) has outcompeted the condition of 4°C as all the different Nostocalean strains survived and revived pretty well (photoplate-23).

It was reported that in general, akinetes and heterocysts showed a higher level of viability from a long term preservation than the vegetative cells (Watanabe et al., 1984). However, several parameters are generally considered very important in cryopreservation, including the choice of the cryoprotectant, cryoprotectant concentration, freezing rate, physiological status of the culture and thawing procedure (Apt and Behrens, 1999). Freezing a suspension of living cells resulted in several events that can be detrimental to the viability of these cells (Mazur, 1977). A primary mode of lethal cell injury was the formation of
intracellular ice (Day et al., 1998). Cryoprotectants are added to the suspension of cells to help minimize the damage experienced during freezing.

A wide variety of cryoprotectants have been tried, including DMSO, glycerol, methanol, polyvinylpyrrolidone, proline, propylene glycol, ethylene glycol, sorbitol, glucose, sucrose, dextran and betaine (Canavate and Lubian, 1995; Andersen, 1996; Kono et al., 1997). Glycerol, DMSO and methanol are the most widely used cryoprotectants and each has been shown to give good success rates (Beaty and Parker, 1990; Canavate and Lubian, 1995). Skimmed milk, bovine serum albumin (BSA) and glycerol, which are often used as cryoprotectant for bacterial preservation, are not effective for cyanobacteria.

Likewise, DMSO was known as the most effective cryoprotectant for algae (Simione and Brown, 1991) which supports our results as all the 06 strains revived very well. Cryoprotectant toxicity may be one factor that is responsible for the cell damage on freezing and thawing, as the cells remain exposed to cryoprotectant during much of the freezing and thawing processes (Day et al., 1998) which could be the reason for the poor revival rate of the 06 strains cryopreserved using cryoprotectant glycerol. To examine the changes of cultural characteristics by cryopreservation, the growth of biomass in liquid medium after preservation treatment was carried out for chl-a which is an indicator of growth and found satisfactory with both the cryoprotectants.

Growth rates of immobilized cells are generally found to be lower than those of corresponding free cell cultures (Bailliez et al., 1985; Robinson et al., 1985; Abdel Hameed, 2002). An opposite trend was demonstrated by Chevalier and de la Noue (1985a). They reported that the maximum growth rate observed during the exponential phase was essentially the same for immobilized and free cells. Similar observations were reported (Tam et al., 1994; Lau et al., 1998a, b; Kobbai et al., 2000). However, Rai and Mallick (1992) reported a higher final yield for alginate immobilized Anabaena and Chlorella after 15 days in growth.
medium compared to free living cells and this is in support with our results where the cultures: *Anabaena* sp. BTA650, *Nostoc* sp. BTA197, *Rivularia* sp. BTA510, *Scytonema hofmanni* BTA124, *Anabaena* sp. BTA281 and *Calothrix* sp. BTA265 immobilized and stored at 18±2°C showed high yield of chlorophyll-a (fig.10).

The present investigation indicates that DMSO was better cryoprotectants compared to glycerol. The technique of cryopreservation can therefore be of help for preservation of cyanobacterial cultures for longer period without losing viability when compared to the maintenance or preservation of cyanobacterial cultures in cultural conditions which required frequent subculturing of the strains.